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Properties and mechanistic aspects of newly found redox enzymes from anaerobes suitable for bioconversions on preparatory scale

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Abstract - Anaerobes or facultative anaerobic bacteria show interesting new types of reversible carbon-hydrogen bond forming enzymes in high activities, which are often not NAD(P)(H) dependent. Their substrate specificity is very broad and nevertheless, their stereoselectivity very high. Some properties and results with enoate reductase, viologen accepting 2-hydroxycarboxylate-oxidoreductase (HVOR), and aldehyde oxidoreductase, reducing non-activated carboxylic acids to aldehydes, will be described. Due to the rather ubiquitous occurrence of viologen accepting pyridine nucleotide oxidoreductases (VAPOR) also yeasts can be used for reductions in electrochemical cells with efficiencies much higher than with carbohydrates.

INTRODUCTION

Purified redox enzymes or microbial cells have been used as stereoselective redox biocatalysts for many years. The reducing enzymes applied were usually pyridine nucleotide dependent (ref. 1,2). New enzymes for this purpose were found and introduced for preparative work (ref. 2,3). The application of isolated pyridine nucleotide dependent enzymes on a preparative scale requires coenzyme regeneration. Various methods have been developed (ref. 4,5). The conditions for technical applications are known, e. g. by the work of Kula and Wandrey (ref. 5). As far as cells are concerned, mostly various types of yeasts have been applied (ref. 6,7). Methods have been studied for controlling the enantioselectivity of reductions with baker's yeast (ref. 8).

Some time ago we became interested in redox enzymes occurring in anaerobes. We assumed that during evolution, in the absence of noticeable concentrations of dioxygen in the earth atmosphere redox enzymes may have been evolved creating carbon-hydrogen bonds, which are not stable in the presence of oxygen. It was known, that strict anaerobes contain redox enzymes such as hydrogenases, reversible carbon monoxide dehydrogenases, nitrogenases and others, which are hardly found in aerobes. The presence of carbon-hydrogen forming enzymes was screened by using resting anaerobes such as clostridia or anaerobically grown facultative anaerobes as catalysts for the reduction of various unsaturated compounds such as ketones, oxo acids, 2-enoates, 2-enones and non-activated carboxylic acids, at the expense of dihydrogen, formate or carbon monoxide. We found that the C-C double bonds of non-activated 2-enoates, the C-O double bonds of oxo acids and aldehydes or even non-activated carboxylic acids can be reduced by non-pyridine nucleotide dependent enzymes with dihydrogen, formate or carbon monoxide. It turned out that interesting unknown redox enzymes exist in strict and facultative anaerobes, if the latter are grown under strict anaerobic conditions. Some of the newly detected enzymes together with suitable methodologies are of interest for preparative work.

If whole cells or crude extracts are used we apply a measure for the efficiency of the microbial bioconversions, which is defined as follows:

Productivity number PN = mmol product/biocatalyst (dry weight) kg x time (h)

The newly found enzymes and procedures, which we developed can be characterised as follows (ref. 9-11):

(i) By using whole cells, productivity numbers are obtained, which are typically one and sometimes up to three orders of magnitude higher than those of conventional

methods with yeasts and carbohydrates as electron donors. That means, that 100-200 g of product can easily be prepared with normal laboratory equipment.

(ii) New types of reactions are catalysed by newly detected enzymes. The later described reduction of non-activated carboxylic acids in water shows a reaction, which is not known by chemical means. Usually resting cells of anaerobes such as clostridia or anaerobically grown facultative organisms such as *Proteus vulgaris* are applied.

(iii) As electron donors hydrogen gas, formate, carbon monoxide, or the cathode of an electro-chemical cell rather than glucose are used. Also, yeasts can be used very effectively in electro-chemical cells increasing their productivity numbers 1-2 orders of magnitude (ref. 12) or they can be coupled to hydrogen gas (ref. 13). This is made possible by the enzymic reduction of NAD to NADH with reduced MV. Under anaerobic conditions the specific VAPOR activity of mitochondria is about 10-20 times higher than the oxidation of succinate or NADH by oxygen (ref. 14).

(iv) Most of the oxido-reductions, which will be described are not pyridine nucleotide dependent, but - and that is very important -the enzymes accept single electrons from, or deliver single electrons to artificial mediators. Mostly the natural cosubstrates are not known but artificial mediators such as viologens of different redox potential work very well. This is important because it opens the way for various electro-microbial redox reactions. A short review appeared recently (ref. 15). The above mentioned higher efficiencies are observed regardless whether non-pyridine nucleotide dependent or pyridine nucleotide dependent enzymes are involved in the substrate reduction. Especially the anaerobes have rather high VAPOR activities (ref. 9, 16). Reduced viologens can also be prepared with dihydrogen gas by modified palladium, platinum or nickel catalysts, which in this modified form do not reduce carbon-carbon or carbon-oxygen double bonds (ref. 13).

(v) Mediators for these new enzymes may be methylviologen (MV) and benzylviologen (BV), which are commercially available. Their redox potential E'_o is -443 and -360 millivolts, respectively. These values are for 1,1',2,2',-tetramethylviologen (TMV) about -555 mV and for carbamoyl methylviologen (CAV) -295 mV. These differences have drastic effects on the equilibrium constants of redox reactions. If pyruvate is reduced to lactate at pH 6.0 with $MV^{+\cdot}$ the equilibrium constant is about $3x10^{10}$. At pH 8.5 in the presence of $CAV^{+\cdot}$ this value changes to 3.5. That means, there are 10 orders of magnitude difference. Furthermore, the viologens can be applied in electro-microbial or electro-enzymic reactions in special ways. The N,N'-diaminopropyl-4,4'-bipyridinium dication (DAPV) can be bound with one amino group to the carboxyl group at the surface of cathodes (oxidised graphite) and the other amino group can be used for the covalent fixation of an enzyme (ref. 17) or the enzyme may be in solution and only the mediator is fixed to the carbon electrode (ref. 18). Also Co-sepulchrate is an interesting mediator. It can be applied instead of viologens for the following reductions (ref. 19): enoates with *C. tyrobutyricum*, 2-oxo acids to 2R-hydroxy acids with *Proteus vulgaris*, esters of 3-oxo-butyric acid or derivatives such as ethyl 3-oxo-4-chloro-butyrate with *C. kluyveri* to the corresponding chiral 3-hydroxy acids, carboxylic acids to the corresponding alcohols with *C. thermoaceticum* or with various VAPORs it can be used for the electro-chemical formation of NAD(P)H.

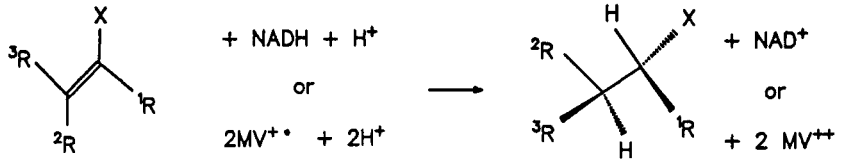
(vi) The microorganisms or viologen accepting enzymes together with described methodologies are very suitable for the preparation of stereoselectively hydrogen labelled products (ref. 20).

ENOATE REDUCTASE

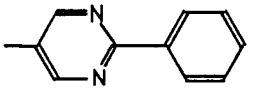
The first new enzyme very suitable for preparative work, which we found in our studies is enoate reductase from *Clostridium tyrobutyricum*. This enzyme shows a surprising broad substrate specificity for 2-enoates (ref. 21). The same seems to be true for the enzyme from *C. kluyveri* (ref. 22). In other clostridia in which enoate reductase seems to play an essential role in the catabolism of amino acids there is a rather strict substrate specificity (ref. 23). The stereochemical course of the enoate reductase has been determined on various occasions. Only a strict stereospecific trans hydrogen addition has been observed (ref. 21, 24, 25). Six different reactions are catalysed by enoate reductase (ref. 21): Those interesting from a preparative point of view are shown in Scheme 1.

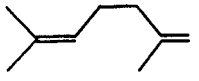
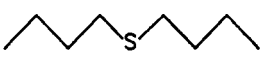
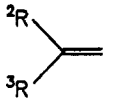
Additional double or triple bonds isolated or in conjugation to the double bond in 2-position are not reduced (ref. 24, 25). Suitably substituted racemic allene carboxylates are stereospecifically reduced to a mixture of E and Z forms (ref. 9, 21).

Scheme 1 REACTIONS of ENOATE REDUCTASE



X = COO^-, CHO; ^1R = Me, Eth, halogen, OMe, SMe, NHCHO.

^2R almost no limitation from CH_3 to C_7H_{15}  ,  ,

 ,  ,  , but also ROOC. ^3R should not be bulky.

The non-reversible reduction of enoates can be carried out by NADH or more effectively with reduced methylviologen. In the latter case reactions can be conducted in an electro-chemical cell with isolated enzyme (ref. 26) or cells of *C. tyrobutyricum*. For further examples see (ref. 9,10). 2-Enoates with chlorine or bromine in 3-position cannot be converted to the corresponding 3-halogenocarboxylates since the halogen is enzymicly eliminated and the resulting halogen-free 2-enoate is further reduced (ref. 21). It is also possible to reduce 2-enals to the corresponding saturated aldehydes. In contrast to the reduction of 2-enoates this reaction is reversible. That means, saturated aldehydes can be dehydrogenated. Dichlorophenol-indophenol is suitable as an electron acceptor. Differing from the reaction with 2-enoates chiral, saturated aldehydes are racemised by enoate reductase. If they are immediately reduced further to the corresponding alcohols practically complete enantioselectivity can be reached (ref. 27). Further informations on the structure, on reactions and kinetic as well as mechanistic aspects of enoate reductase are described in (ref. 21).
Preparative reductions are mostly conducted with resting cells of *C. tyrobutyricum*. For some more details see (ref. 9, 21, 22).

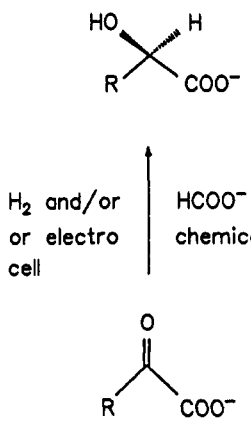
2R-HYDROXYCARBOXYLATE-OXIDOREDUCTASE

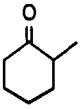
2-Hydroxycarboxylate viologen oxidoreductase (HVOR) from *Proteus vulgaris*, a membrane bound enzyme, which we detected some time ago reduces an extremely wide variety of 2-oxocarboxylates to the corresponding 2R-hydroxycarboxylates at the expense of reduced methyl or benzyl viologen. The reaction rates with the various substrates are usually on the same order of magnitude. The productivity numbers under optimal conditions in electro-chemical cells with the most effective *P. vulgaris* cells should be in the range from 50 000-400 000 (ref. 28). Under very simple conditions the productivity numbers still reach values in the range from 3 000-50 000. Neither NADH nor NADPH is a cosubstrate (ref.9-11). Scheme 2 shows substrates.
We developed simple procedures by which resting cells of *P. vulgaris* in the presence of 1 mM viologens reduce many different multifunctional 2-oxocarboxylates on multi gram scales to 2R-hydroxycarboxylates, which are chirally pure within the limits of detection (Scheme 2). Resting cells of *P. vulgaris* possess hydrogenase and viologen dependent formate dehydrogenase for the regeneration of the reduced viologen at the expense of hydrogen gas and/or formate (ref. 29) and showed that the products can be used for creating further chiral centres by chemical reactions. (ref. 30).
HVOR is also able to dehydrogenate stereoselectively 2R-hydroxycarboxylates if an oxidised viologen of suitable redox potential at pH values of about 8.5 is used (ref. 31). So the reaction can be formulated as follows:

$$RCOCOO^- + 2\ V^{+\cdot} + 2H^+ \rightleftharpoons 2R\text{-}RCHOHCOO^- + 2V^{++}$$

Under strictly anaerobic conditions wet packed cells of *P. vulgaris* at -15 °C or freeze dried cells at room temperature are a rather stable biocatalyst loosing less than 10 % of its activity during 1-2 years.
Chiral 2-hydroxycarboxylic acids are valuable synthons. This is especially true if they contain additional functional groups, which by chiral induction can be converted to further chiral centres in diastereoselective chemical reactions. Therefore, we prepared various 2R-hydroxy acids such as 3-enoic-, 3,5-dienoic-, 4-oxo- 3R,S-hydroxy acids besides some others on a scale up to 0.12 mol from the corresponding 2-oxo acids with *P. vulgaris* and hydrogen gas and/or formate as electron donors. With the exception of the 2-hydroxy-4-oxo acids it could be proved that the enantiomeric excess is >97%. For the 4-oxo derivatives this enantiomeric excess can be assumed (ref. 29). The yields of isolated products are high because they were isolated from rather small amounts of biocatalyst and low buffer concentrations. Product concentrations in the range of 0.1-0.24 M were obtained. For 1 mmol of product formation in 15-20 h about 20-40 mg (dry weight) of *P. vulgaris* cells are necessary. Racemic 2-oxo acids with a chiral carbon atom in 3-position show only rather low differences in their reduction rate. The use of the aforementioned 2R-hydroxy-3-enoic acids for creating addition products with peracids, osmium tetroxide, methylrhenium trioxide, N-bromo succinimide, bromine or iodine have been studied. High and very high diastereomeric excess values have been observed. The latter is especially true for the preparation of 2S,3R,4S-2-hydroxy-3-halogeno-butyrolactones. A diastereomeric excess of more than 96 % has been observed for the 3-iodolactones (ref. 30). As mentioned above by using oxidised CAV at pH 8.5, stereochemically pure 2S-hydroxycarboxylates together with the 2-oxocarboxylates can be obtained from a racemic mixture of 2-hydroxycarboxylates (ref. 11, 31).

Scheme 2 REDUCTIONS of 2-OXO-ACIDS WITH *PROTEUS VULGARIS* LEAD TO 2R-HYDROXYCARBOXYLATES



Types of residues R	Examples
(i) unbranched and branched alkyl	R = from CH_3 to at least C_6H_{13} R = $(CH_3)_3C$; (R,S)-$C_2H_5CHCH_3$
(ii) OH group in 3-position	R = (R,S)-C_3H_7CHOH
(iii) One or two conjugated carbon carbon double bonds	R = R'  with aliphatic or aromatic R' also R"OCH_2-
(iv) Additional CO group	R = $C_6H_5COCH_2$,  and others
(v) $^-OOC(CH_2)_n$	n = 1 to at least 3

So far no satisfying reaction with R = R'CHX- (X = Cl or Br)

During our efforts to improve the growth medium for *P.vulgaris* aiming at high specific activities of this membrane bound enzyme it turned out that HVOR is a molybdenum requiring enzyme. In the meantime growth conditions were developed, which show in the crude extract more than 30 U of HVOR, which corresponds to about 4-5 U for the back reaction (unpublished).
HVOR is an interesting new type of a molybdo enzyme because to the best of our knowledge no other enzyme seems to be known, which reduces a carbonyl group without being pyridine nucleotide dependent. Most molybdo enzymes contain flavins or hem systems as additional prosthetic groups. That does not seem to be the case for HVOR. It contains a molybdenum cofactor known as MoCo since pterine-6-carboxylic acid can be obtained by oxidising the purified enzyme with permanganate. It consists of an α,β-structure with apparent masses of 80 and 64 kDa of the subunits. Sequencing of the amino end of both subunits shows no similarity with other known protein sequences. The membrane bound enzyme oligomerises to species of more than 600 kDa. Purified enzyme shows activities of more than 1000 U/mg protein (unpublished). (One

unit reduces per min 1 μmol 2-oxo acid to the corresponding 2R-hydroxy acid.)
The purified enzyme converts a 1:1 mixture of a 2R-hydroxy acid and a 2-oxo acid with different carbon skeletons to a mixture containing two 2-hydroxy- and two oxo acids in an almost 1:1:1:1 ratio. That means, for this reaction a coenzyme does not seem to be necessary. In deuterium oxide the α-hydrogen of 2R-hydroxy acids is exchanged by the purified enzyme.

CARBOXYLIC ACID REDUCTASES

Recently we observed the capability of *Clostridium thermoaceticum* and *C. formicoaceticum* to reduce non-activated carboxylic acids at the expense of carbon monoxide or formate to the corresponding alcohols. In the presence of 1 mM methylviologen the reduction rate can be accelerated (ref. 32-34).
In studying the enzymology of the carboxylic acid reduction we detected in *C. thermoaceticum* and *C. formicoaceticum* tungsten containing enzymes, which reduce non-activated carboxylic acids to the aldehydes by reduced viologens (ref. 34-36). The reaction

$$RCOOH + 2V^{+\cdot} + 2\,H^{+} \rightleftharpoons RCHO + 2\,V^{++} + H_2O$$

is reversible i. e. the carboxylic acid reductase can also be called a reversible aldehyde oxidoreductase. Pyridine nucleotides are not involved in this reaction. The natural electron carriers are not known yet. The reduction of the aldehydes to the aforementioned alcohols are catalysed by other enzymes. The properties of the aldehyde oxidoreductase from *C. thermoaceticum* (ref. 34, 36) are astonishingly different from that isolated from *C. formicoaceticum* (ref. 35) but both enzymes contain tungsten and a pterin together with iron sulphur clusters.
The substrate specificity of the reductase from *C. thermoaceticum* is very broad. Aliphatic as well as aromatic mono- and di-carboxylic acids are reduced. Not as many substrates have been studied with the enzyme from *C. formicoaceticum*. Again there are clear-cut differences but also similarities. Surprising are the extremely high K_m values for 4-hydroxybenzoic acid and to a lesser degree of 3-hydroxybenzoic acid. The corresponding methoxy- or halogeno derivatives have K_m values in the range of 1.2 to 6.3 mM.
A drastic difference shows the dependence of the reaction rate on the pH. The pH-dependence for the enzyme from *C. thermoaceticum* is near 4.5 and its dependence on the pK_a value of the acid leads to the conclusion that the non-ionised carboxylic acid rather than the carboxylate anion is the substrate. The pH optimum of the enzyme from *C. formicoaceticum* is 1.5 units higher. This is reflected in the redox potentials of the artificial mediators necessary to carry out the reductions with the purified enzymes. A negatively charged group like the carboxylate anion accepts electrons less easily than the neutral carboxyl group. For the reduction with the *C. thermoaceticum* enzyme reduced CAV is sufficient. The enzyme from *C. formicoaceticum* needs reduced MV or TMV.
It is possible to reduce many different acids to alcohols (ref. 32 and unpublished work). Also pyridine- and chlorinated pyridine carboxylic acids can be reduced to the corresponding hydroxymethyl pyridines.
Very recently Mukund and Adams (ref. 38) described a tungsten-iron-sulphur containing aldehyde ferredoxin oxidoreductase from the hyperthermophilic *Pyrococcus furiosus*. The purified enzyme oxidises glyceraldehyde and other aldehydes at the expense of oxidised ferredoxin. A new "pyroglycolytic" pathway is postulated in which 2-oxo-3-deoxygluconate is split to glyceraldehyde and pyruvate. In a ferredoxin dependent reaction the aldehyde is dehydrogenated to glycerate. Reductions of carboxylic acids were not reported.

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